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Mitochondrial DNA as a potential translational biomarker of mitochondrial dysfunction in drug induced toxicity studies.

Afshan N Malik

King's College London, Diabetes Research Group, Faculty of Life Sciences and Medicine, London, UK

CONTENTS

1. ABSTRACT

Mitochondria contain their own extra-nuclear genome in the form of mitochondrial DNA (MtDNA), a small circular DNA molecule which is present as multiple copies within cells. MtDNA levels can change in response to physiological conditions and oxidative stress, and MtDNA copy numbers relative to nuclear genome copy numbers have been proposed as a biomarker of mitochondrial dysfunction. In the last decade, increasing number of studies have reported alterations in MtDNA in body fluids and tissues from human populations in both common diseases and in response to drug/environmental toxicity. Changes in MtDNA levels have been reported in prevalent diseases such as diabetes and its complications, cancer, HIV, neurodegenerative diseases, cardiovascular disease, and also correlate with patient response to chemotherapy, anticancer drugs and HIV treatment. Additionally, cell free MtDNA has emerged as a potential danger associated molecular pattern (DAMP) molecule with the potential to induce inflammation, and has been linked to increased risk of cardiovascular disease, sepsis and mortality in intensive care unit patients. However, although drug-induced mitochondrial dysfunction is accepted as a key issue to resolve in order to prevent drug toxicity, few studies have used MtDNA as a potential indicator of drug toxicity during drug design and development. Furthermore, methodological issues in accurately measuring MtDNA have resulted in conflicting data for the same disease in different populations, and physiological levels of MtDNA in human and animal model body fluids, tissues and organs remain to be determined . The accurate measurement of MtDNA requires careful assay design, avoidance of co-amplification of nuclear pseudogenes, and clarification of whether cellular and cell free MtDNA is being measured, and there is a clear need for the improvement of methodology. However despite these issues, we suggest that that the determination of the impact of drugs on cellular and cell free MtDNA could provide an extremely useful indicator of drug induced toxicity, and could lead to its utilisation in parallel with existing methodologies in the initial drug development and testing phase as well as in follow up clinical trials to test drug efficacy and patient response.

2. INTRODUCTION

Mitochondria are double membrane organelles which produce the majority of cellular energy in eukaryotes in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). As well as energy production, mitochondria have many other crucial cellular functions, including the regulation of intracellular calcium homeostasis and apoptosis (Wojtczak and Zablocki, 2008). Because of their role in energy production and other key cellular functions, damage to mitochondria can have a serious impact on the health of cells and tissues and can result in a variety of diseases (Wallace, 1999). In recent years it has become widely accepted that systemic damage to mitochondria, often termed acquired mitochondrial dysfunction, is involved in many common human diseases (Michel et al., 2012, Wallace, 1999, Malik et al., 2013) as well as in drug induced toxicity (Dykens and Will, 2008) leading to a growing interest in developing biomarkers of mitochondrial health.

Mitochondrial energy production is carried out within the double membrane of mitochondria via electron transport through a complex of proteins known as the electron transport chain. During mitochondrial ATP synthesis, electron leakage from the electron transport chain can lead to the production of reactive oxygen species (ROS), which in normal conditions is involved in signalling, however excess ROS can lead to oxidative stress. In normal healthy cells mitochondria are present as an interconnected network or several networks rather than the old fashioned view of solitary organelles (Bereiter-Hahn et al., 2008). Cellular mitochondrial content is regulated via mitochondrial biogenesis and degradation of mitochondria via mitophagy. The mitochondrial mass reflects the bioenergetics requirements of the host cell, and can vary from tens to thousands of mitochondria per cell. The number of mitochondria in different cell types therefore varies widely, for example a brain cell may have around 2000 mitochondria (Uranova et al., 2001), a white blood cell may have less than a hundred (Selak et al., 2011) and oocytes may contain several hundred thousand mitochondria (Duran et al, 2011, Piko and Matsumoto, 1976). The number of mitochondria in a particular cell type also can vary in response to

environmental and physiological factors, for example cellular redox balance or signalling pathways (Michel et al., 2012, Rodriguez-Enriquez et al., 2009). Damage to mitochondria, once it exceeds a threshold, can affect a range of important cellular functions and can contribute to the development of a large number of diseases (Michel et al., 2012; Wallace, 1999). Cells and tissues with high bio-energetic needs and consequently high mitochondrial mass are particularly sensitive to the impact of mitochondrial damage. Consequently, there is a strong need for translational biomarkers which can be used for early detection of potential mitochondrial dysfunction before irreversible damage to susceptible cells, tissues and organs takes place (Figure 1).

Mitochondria are the only cytosolic organelles in eukaryotes that contain endogenous DNA outside of the nucleus. Mitochondrial DNA (MtDNA) is normally located within mitochondria as a small, circular, extra-nuclear genome. Each mitochondrion can contain multiple copies of MtDNA (Bogenhagen, 2011, Falkenberg et al., 2007), and since cells contain many mitochondria, MtDNA is present as multiple copies within cells. The amount of cellular MtDNA has been shown to correlate with mitochondrial function and OXPHOS activity (Hock and Kralli, 2009, Williams, 1986) and this has led to studies using its quantity as a determinant of mitochondrial activity.

In the last decade, numerous studies have shown that MtDNA levels are altered in disease conditions in tissues and in circulation, and additionally MtDNA has emerged as a danger associated molecular pattern (DAMP) with the potential to induce inflammation (Zhang et al., 2010). However, to date, few studies have attempted to use alterations in cellular or cell-free MtDNA in studies of drug toxicity. In this chapter, I propose the potential of using MtDNA levels in drug toxicity studies, both in–vitro and animal studies, as well as in clinical studies, to monitor the effects of pharmacological compounds on mitochondrial health.

3. THE MITOCHONDRIAL GENOME.

The human mitochondrial genome is 16,569bp long, contains 37 genes, encoding 13 proteins and 24 transfer RNA and ribosomal RNAs crucial to mitochondrial function. The remaining mitochondrial proteins which are required to make functional mitochondria are coded for and transcribed from the nuclear genome, with resultant transcripts being translated into proteins at cytosolic ribosomes and transported into mitochondria for assembly (Scheffler, 2008). Mitochondria contain >1000 different proteins, some of which show tissue specific profiles (Johnson et al., 2007, Smith et al., 2012). The correct functioning of all 37 genes encoded by the mitochondrial genome is crucial but to make functional mitochondria and a functional electron transport chain. This is because MtDNA encodes 13 protein subunits crucial for the mitochondrial OXPHOS machinery as well as various RNAs required for mitochondrial protein synthesis. These components, together with nuclear encoded proteins, result in the assembly of functional mitochondrial mass in cells and allow mitochondrial function and energy production in the form of ATP. Therefore, MtDNA has a crucial role in cells by acting as a template for both transcription and replication to generate functional mitochondria. Bio-energetically active tissues such as brain, heart, kidney and muscle with a high mitochondrial content thereby can contain hundreds of thousands of copies of MtDNA per cell, whereas other tissues and cells such as blood cells with less mitochondrial mass contain considerably less MtDNA per cell (Mercer et al., 2011, Fernandez-Vizarra et al., 2011, Malik et al., 2016).

Under normal conditions, the amount of MtDNA can change in response to cellular physiological signals, with cells maintaining a balance between MtDNA replication and transcription to allow mitochondrial biogenesis as needed. However, in certain disease conditions, this relationship breaks down and cellular MtDNA content may increase in response to oxidative stress but transcription and translation of MtDNA are blocked, leading to increased cellular MtDNA which in time may become damaged. MtDNA damage could comprise of mutations, deletions and oxidation. The integrity and amount of MtDNA present in cells can impact on mitochondrial function (Madsen-Bouterse et al., 2010, Czajka et al., 2015). MtDNA

damage can have downstream effects on cellular health, causing defects in the OXPHOS machinery, cellular signalling, and subsequently leading to oxidative stress, an energy deficit and eventually cell death. MtDNA damage may thus lead to release of cellular content including MtDNA into circulation, and if systemic mitochondrial dysfunction is present, the release of large amounts of cellular contents and MtDNA may compromise the body's capacity to clear circulating MtDNA.

Some of the key differences between the human mitochondrial and nuclear genomes are listed in Table 1. Eukaryotic DNA in the nuclear genome is organised as chromosomes, large double stranded linear molecules stored as highly packaged and compact structures and are stored as chromatin, a DNA-histone protein complex. In contrast, MtDNA exists as a small circular double stranded DNA molecule organised into a nucleoprotein with the transcription factor A (TFAM) protein, and termed a nucleoid. Nucleoids are found associated with the inner mitochondrial membrane (Bogenhagen, 2011, Falkenberg et al., 2007) and individual mitochondria can contain several copies of the mitochondrial genome (Navratil et al., 2007; Veltri et al., 1990). The differences between the mitochondrial and nuclear genome (table 1) can significantly impact on methodology used for the measurement of MtDNA, and are discussed in more detail below (see section 5).

4. IS MITOCHONDRIAL DNA A USEFUL BIOMARKER OF MITOCHONDRIAL DYSFUNCTION

The amount of MtDNA in a cell could provide a major regulatory point in mitochondrial activity, as the transcription of mitochondrial genes is proportional to their copy number (Hock and Kralli, 2009, Williams, 1986). Indeed, MtDNA has been widely utilised as an indicator of cellular mitochondrial content. We previously proposed the hypothesis that MtDNA content measured as Mt/N (mitochondrial to nuclear genome ratio) is a biomarker of mitochondrial dysfunction (Figure 2, Malik and Czajka 2012).

The premise of this theory is that the Mt/N value of a particular cell type changes in conditions of stress such as redox imbalance or other altered signalling. The initial response to increased cellular stress

would be an adaptive response where Mt/N values would increase as a result of increased mitochondrial biogenesis. In conditions of persistent oxidative stress, alterations in Mt/N may represent a mixture of intact and functional mitochondrial genomes as well as damaged MtDNA fragments which have not been properly removed. Oxidative stress may eventually lead to the depletion of Mt/N alongside mitochondrial dysfunction resulting from damaged MtDNA and proteins. Accumulation of damaged MtDNA in the cell may lead to an inflammatory response as MtDNA is un-methylated and resembles bacterial DNA (Figure 2).

Oxidative stress is a common feature in many diseases including diabetes complications, cardiovascular disease, neurodegenerative disease, cancer, renal disease and others (Halliwell and Gutteridge, 2007). Free radicals, also known as reactive oxygen species (ROS) are produced as a side product of using oxygen for energy production, and are highly reactive molecules with unpaired electrons. It has been estimated that approximately 5% of the oxygen being used in the body turns into ROS, as a consequence of electron leakage from the electron transport chain during oxidative phosphorylation (Adam-Vizi and Chinopoulos, 2006; Halliwell and Gutteridge, 2007; Turrens, 2003). With the exception of phagocytes, cells produce more than 95% of their intracellular ROS via the mitochondrial electron transport chain. Most cells are well equipped to deal with intracellular ROS as they have endogenous antioxidant systems such as glutathione peroxidise, catalase, and superoxide dismutase (Nohl, 1991; Nordberg and Arner, 2001). These highly abundant cellular proteins, present in most cells, can sequester ROS by accepting electrons and becoming oxidised, and are usually recycled by donating their electrons to chains of acceptors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Rydstrom, 2006). The cell's metabolic performance is closely related to its antioxidant response, and NADPH levels are central to the activity of many antioxidants (Kirsch and De Groot, 2001). Despite these endogenous antioxidant systems, when chronic ROS production occurs, the cell's ROS levels can exceed their detoxification and cause a shift in the redox balance. Free radicals which escape

the cells' antioxidant response can oxidise proteins, lipids and DNA molecules within the cell, leading to altered properties and cellular damage. Many common drugs cause mitochondrial oxidative stress (reviewed by Mehta et al., 2008), and many common diseases such as diabetes and its complications, cancer, neurodegenerative disorders as well as ageing have been shown to have redox impairment (Halliwell and Gutteridge, 2007; Wallace, 1999; Ying, 2008).

The mitochondrial life cycle controls cellular mitochondrial mass, through both mitochondrial biogenesis, the synthesis of new mitochondria, and mitophagy, the degradation and removal of damaged mitochondria. Evidence indicates that both biogenesis and mitophagy may be impaired in conditions of oxidative stress. Abnormal signalling results in an adaptive response through enhanced production of mitochondria (Michel et al., 2012). Reduced removal results in the accumulation of damaged mitochondria (Kim et al., 2007) as is the case for diabetes where blockage of the electron transport chain at complex III results in accumulation of excess ROS (Giacco and Brownlee, 2010; Newsholme et al., 2007). As mitochondrial DNA is located close to the source of ROS production, the DNA itself can become damaged resulting in accumulation of deletions and mutations (Bohr, 2002; Croteau and Bohr, 1997; Indo et al., 2007).

Accumulation of damaged MtDNA, alongside its ineffective clearance, may result in its release from damaged mitochondria and cells and cause a chronic innate inflammatory response. In such a scenario, MtDNA could contribute directly to pathology because unlike eukaryotic nuclear DNA which is often methylated at CpG motifs within DNA, MtDNA DNA is largely un-methylated like bacterial DNA. Un-methylated DNA is known to cause immune responses via the intracellular Toll like receptor (TLR) 9 (Barbalat et al., 2011; Sparwasser et al., 1997). Injection of oxidised MtDNA directly causes inflammatory arthritis in mice (Collins et al., 2004). Zhang et al., 2010 showed that circulating MtDNA levels were markedly increased in trauma patients and provided a mechanistic explanation for this observation by showing that MtDNA could directly activate human neutrophils via TLR 9 (Zhang et al., 2010).

Accumulation of MtDNA in the cytosol of cardiomyocytes resulted in heart failure in a mouse model where the normal process of degradation of damaged mitochondrial DNA had been disrupted (Oka et al., 2012). Therefore, altered MtDNA levels may elicit an increased immune response resulting in chronic inflammation and oxidative stress, thus contributing directly to pathogenesis. In parallel, loss of cellular MtDNA would cause reduced mitochondrial function and a bioenergetic deficit, which would further impair the cell's ability to repair cellular damage.

According to our hypothesis (Figure 2), in conditions of oxidative stress, the transcriptional and replication machinery of mitochondrial biogenesis will be up-regulated as a maladaptive response resulting in increased mitochondrial biogenesis via replication of the mitochondrial genome (Malik & Czajka, 2013)). There are some studies in the literature supporting the view that ROS can lead to increased mitochondrial biogenesis. In human endothelial cells, homocysteine-induced ROS resulted in increased expression of TFAM and NRF-1 genes, and this effect was abolished by antioxidant treatment (Perez-de-Arce et al., 2005). In human lung fibroblasts, following treatment with hydrogen peroxide to induce oxidative stress, there was an increase in mitochondrial mass and MtDNA copy number (Lee et al., 2000). Up-regulation of transcriptional machinery was shown to be protective against oxidative stress, for example over-expression of recombinant TFAM *in-vitro* and *in-vivo* can stimulate mitochondrial biogenesis and reduce oxidative stress (Thomas et al., 2011). Lee and Wei proposed that mild oxidative stress leads to increased mitochondrial biogenesis and copy number, and suggested that the stress response of cells in terms of mitochondrial copy numbers and biogenesis could be key in terms of the life or the death of the cell and should be further investigated (Lee and Wei, 2005). Moreover, in a study of 156 healthy subjects, ranging from the ages of 25 to 80, it was found that MtDNA content in leucocytes was higher in volunteers with increased levels of oxidative stress (Liu et al., 2003).

We recently showed that growth of primary human renal glomerular mesangial cells in high glucose led to a rapid increase in cellular MtDNA in parallel with increased oxidative stress (Czajka et al 2015) and that these changes preceded other measures of mitochondrial dysfunction. Interestingly, the increased MtDNA was not functional since MtDNA encoded mRNAs were not up-regulated in parallel. Instead, the MtDNA was damaged and there was up-regulation of the TLR9 pathway in parallel (Czajka et al., 2016). These data support the hypothesis that oxidative stress can lead to early and detectable changes in MtDNA. Interestingly, we further showed that the MtDNA changes preceded mitochondrial dysfunction, since MtDNA changes were detectable within 24 hours of growth in high glucose whereas cellular respiration remained functional until 8 days (Figure 3). This further supports the view that MtDNA changes take place early on and maybe used as an indicator of mitochondrial dysfunction before damage to cellular respiration takes place. These data also suggest that early changes in MtDNA may cause a cascade of pro-inflammatory responses via the early activation of the TLR9 pathway.

5. METHODOLOGICAL ISSUES FOR MEASURING MITOCHONDRIAL DNA CONTENT

As discussed in more detail below and previously described, disease associated changes in MtDNA content from various body fluids have been reported in a broad range of human diseases, as well as in normal development, fertility, and exposure to environmental factors (Malik and Czajka 2013). The use of body fluids for these studies is an attractive option as tissues and organs cannot easily be accessed, and most published studies have tended to use blood samples. A common method for measuring MtDNA content is to quantify a mitochondrial-encoded gene relative to a nuclear-encoded gene to determine the mitochondrial genome to nuclear genome ratio which we have termed Mt/N (Malik et al., 2009). Earlier studies measuring Mt/N utilised hybridization (Rodriguez-Enriquez et al., 2009; Veltri et al., 1990) whereas more recent studies use real time (Cavelier et al., 2000; D'Souza et al., 2007; Malik et al., 2011) or digital (Masser et al., 2016) quantitative PCR (qPCR), a highly sensitive technique which is fast, adaptable for high throughput and widely available. This has resulted in the utilisation of this

technique in determination of Mt/N in a large number of studies of clinical samples (reviewed in the next section).

MtDNA quantity in the periphery, in circulating peripheral blood cells as well as in cell-free fluid of blood such as plasma, is a highly feasible screening tool for translational studies. However, both increases and decreases in MtDNA have been reported in pathogenic conditions. Currently there is no standard for defining what constitutes an abnormal MtDNA quantity in different sample types, and data from different populations for specific diseases have been inconsistent. Many methodological based issues can significantly alter MtDNA values (Malik et al., 2011, Malik and Czajka 2012, Chiu et al., 2003, Hammond et al., 2003, Kam et al., 2013). These include: 1) Duplication of the mitochondrial genome in the nuclear genome; 2) Use of inappropriate nuclear primers; 3) Dilution bias; 4) Template preparation problems. These problems can lead to serious errors and are likely to be in part responsible for the conflicting data in the literature. Many protocols widely used for MtDNA quantification do not meet the criteria of specificity and reproducibility as they fail to take into account either the co-amplification of nuclear regions with high identity to the mitochondrial genome, or the dilution effect (Malik et al., 2011). Furthermore, many published papers do not give the actual copy numbers and rely instead on relative values, which makes the data more difficult to interpret, especially if the samples being used comprise of both cell free and cellular MtDNA. (For a more detailed discussion of these methodological issues please see Malik and Czajka 2013, Ajaz et al., 2015 and Malik et al., 2016).

6. ACQUIRED MITOCHONDRIAL DNA CHANGES IN HUMAN DISEASES.

The aim of this section is to highlight the growing body of evidence which when considered together, is strongly supports the view that MtDNA is a potentially valuable and currently largely overlooked biomarker for drug toxicity studies. Our focus is on studies reporting changes in MtDNA quantity under disease conditions rather than MtDNA damage or deletions/mutations/haplotypes. The wider

availability of qPCR as a methodology has led to a substantial increase in publications reporting changes in MtDNA content in human body fluids and tissues. Changes in MtDNA content have been described for a wide range of human diseases from cancer to diabetes as well as in development, ageing and exercise. We reviewed the literature and showed that dozens of studies had shown changes in MtDNA in a large number of diseases (Malik & Czajka, 2013). Since then the number of studies reporting changes in MtDNA in disease has risen even more sharply.

In the cancer field, altered MtDNA levels have been observed in peripheral blood cells, saliva, tumour tissues, and other body fluids in numerous studies (reviewed in Malik and Czajka 2013) leading us to suggest that control of MtDNA copy number may be dys-regulated in cancer. Altered MtDNA levels were proposed to contribute to the risk of cancer in meta-analysis of numerous studies (Mi et al., 2015, Hu et al., 2016) The dys-regulation of MtDNA levels may have direct consequences for drug therapy response in patients. For example, in one study, the level of MtDNA in breast cancer tissue correlated with patient response to anthracycline chemotherapy, with higher MtDNA levels showing lower drug sensitivity (Hsu et al., 2010) whereas in acute lymphoblastic lymphoma, reduced blood MtDNA after treatment was found to confer increased susceptibility to chemotherapy (Kwok et al., 2011). MtDNA copy number changes are widely described in cancers, and interestingly it has been found that mitochondrial dysfunction induced by chemical depletion of MtDNA or impairment of mitochondrial respiratory chain in cancer cells promotes cancer progression to a chemo-resistant or invasive phenotype. Qu et al., (2015) found that leukocyte MtDNA was an independent prognostic marker of colorectal cancer and could be used to stratify patients for chemotherapy. Chen et al (2016) carried out a meta-analysis of 18 separate studies where MtDNA had been measured in 3961 cases from peripheral blood and/or tumour tissue. Their analysis suggested that increased MtDNA levels in peripheral blood predicted a poor cancer prognosis whereas a better outcome was presented among patients with

elevated MtDNA levels in tumour tissues. Therefore, in the future, selective anticancer therapy development may benefit from using MtDNA alterations to inform drug design.

In the human immunodeficiency virus (HIV) field, the impact of therapy on measureable MtDNA changes is very clearly indicated by evaluation in patients undergoing HIV therapy and strongly linked to the risk of numerous drug induced HIV complications. Antiretroviral therapy (ART), widely used for the treatment of human immunodeficiency virus (HIV), can cause mitochondrial toxicity and many complications. Differences in MtDNA have been shown between the adipose tissue of HIV-infected and ART-treated subjects demonstrating that HIV therapy can impact MtDNA in organs as well as within the periphery showing systemic effects of drug therapy (Buffet et al., 2005). The older ART drugs such as nucleoside reverse transcriptase inhibitors directly affect MtDNA replication and result in tissue specific and organ specific pathologies, and consequently, many studies have reported MtDNA changes in association with drug induced complications in HIV patients. One direct mechanism of MtDNA damage is by the inhibition of DNA polymerase gamma, the enzyme which carries out MtDNA replication and which is particularly sensitive to certain antiviral drugs such as dideoxynucleoside inhibitors. As for various cancers, HIV treatment can cause significant changes in MtDNA and therefore it is very likely that control of MtDNA copy number is compromised as a consequence of HIV infection and/or treatment. *In-vitro* experiments showed that lymphoblast cells with increased MtDNA were more resistant to HIV therapy (Bjerke et al., 2008) suggesting that as in cancer, altered MtDNA could have consequences for HIV therapy.

Changes in MtDNA content have been described for metabolic disorders such as diabetes and obesity, as well as fertility, development and ageing (See Malik and Czajka 2012). We have recently shown that circulating MtDNA levels were independently associated with risk of diabetic nephropathy (Czajka et al., 2015) and in a separate study circulating MtDNA levels and inflammation correlated with risk of diabetic retinopathy, the leading cause of adult blindness (Malik et al., 2015). The dysregulation of MtDNA

content in metabolic diseases suggests that changes in MtDNA content correlate with metabolic changes. Interestingly, the anti-diabetic drug thiazolidinedione (TZD) was shown to result in increased MtDNA in adipose tissue of patients with diabetes, in parallel with increased fat storage and weight gain (Bogacka et al., 2005). Altered MtDNA levels have also been reported in liver disease, chronic renal failure, haemodialysis, and in septic shock where MtDNA is believed to cause systemic inflammatory response syndrome (Malik & Czajka 2012).

MtDNA changes have been reported to correlate with disease in human population based studies of neurodegenerative disease including multiple sclerosis (Blokhin et al., 2008, Varhaug et al., 2016), Parkinson's disease (Pyle et al., 2016), Alzheimer's disease (Lindsley et al., 2012) and Huntington's disease (Peterson et al., 2014) as well as for depression (Kim et al., 2011). MtDNA has become accepted as an activator of both inflammation and the innate immune response, and has been shown to be the cause of organ injury (Oka et al.,2012). Additionally, cell free MtDNA levels in circulation were shown to be a high risk factor for mortality in 2 different studies of patients in intensive care units. Circulating MtDNA levels have been shown to be predictive of mortality in patients admitted to intensive care units (Nakahira et al., 2012) and also correlated with traumatic injury and sepsis (Yamamouchi et al., 2013). MtDNA levels have also shown to be predictive of poor outcome/death in patients who have taken drug overdoses (Mitchell et al., 2014).

An interesting theme emerging from these large number of studies are the reports that suggest that MtDNA levels can correlate with and be an indicator of the effect of exposure to chemicals, drugs or environmental toxins in humans. Occupational exposure to low dose benzene can result in increases in circulating MtDNA and this has been proposed to be a possible cause of increased incidence of leukaemia in this population (Carugno et al., 2012). Exposure to the herbicide Atrazine was shown to result in mitochondrial dysfunction and insulin resistance in an *in-vivo* study (Lim et al., 2009). Using

exfoliated cells from saliva, smokers were found to have increased MtDNA and this increase was independent of age and alcohol intake (Masayesva et al., 2006).

Budnick et al., (2013) evaluated the impact of exposure to pesticides and found that circulating MtDNA showed both alterations in quantity and loss of integrity, leading the authors to propose that MtDNA has the potential to serve as a biomarker for recognizing vulnerable risk groups after exposure to toxic/carcinogenic chemicals. Even in a traditionally genetic disease with a clear nuclear mutation, MtDNA was proposed as a biomarker to follow the progression and treatment response of Huntington's disease by Disatnik et al., (2016). In their model system, they observed both tissue and circulating levels of MtDNA were changing at different stages of disease and in response to treatment.

Therefore a large body of evidence now exists showing that MtDNA levels can be measured in human clinical samples and that disease associated changes can be detected in populations. Indeed the evidence for reported alterations in MtDNA in body fluids of human patients in correlation with many diseases has grown rapidly and in the above section I have only been able to comment on a subset of these. What is clear is that there is widespread interest in using MtDNA as a biomarker in human populations, and with the mounting evidence for a link between patient drug response and circulating MtDNA levels, there is strong potential for the future use of this marker in the field of personalised medicine.

7. CONCLUSIONS AND FUTURE DIRECTIONS

Mitochondrial dysfunction is a key issue in drug development and off-target effects of many drugs may impact on mitochondria. Mitochondrial dysfunction contributes to drug toxicity and adverse side effects via many mechanisms in the cell (Mehta et al., 2008). Although structural similarities of drugs to electron acceptors and donors, assays based on redox dyes, and bioenergetics assays have been successfully employed for screens of mitochondrial effects, such assays do not easily lend themselves for

non-invasive use in human samples. Furthermore, there is a need to develop biomarkers for early detection of mitochondrial dysfunction before tissue and organ damage. Because of its early adaptive response to oxidative stress by increased replication and blocked transcription, MtDNA may provide an indicator of mitochondrial stress prior to other indicators. In addition, MtDNA lends itself to rapid detection via methods such as qPCR and digital PCR, making it an attractive high-throughput biomarker. However, methodology issues have hindered the successful use of MtDNA as a biomarker and led to conflicting and unreproducible findings in some cases. Of particular note in this regard is the presence of nuclear mitochondrial DNA segments (NUMTs) in the nuclear genome which can skew data by co-amplifying nuclear genes when MtDNA levels are being assessed. In addition, assays currently in use seldom distinguish between cell free and cellular MtDNA, the former is of importance as it may be an indicator of inflammation, and the latter is important as it may be an indicator of bioenergetic deficit in the cell. Nevertheless, MtDNA copy number measurements could be successfully utilised in drug toxicity studies. Carefully designed assays which measure absolute copy number and take account of the methodological issues described above could be used in numerous stages of drug development. For example, initial in-vitro screens could utilise target cell lines to define if drugs in development have an impact on cellular MtDNA levels, and if they do, then titration studies could inform potentially safer levels of the drug. In-vivo animal studies could be used to study the systemic effects of potential drugs on MtDNA levels in organs and cells over time, and inform the potential leakage of MtDNA into the periphery which would have implications for inflammation. Once clinical trials commence, MtDNA levels in peripheral blood, compartmentalised as PMBCs for cellular and plasma for cell-free, as well as in urine, compartmentalised as urinary pellet for cellular debris and cell free urinary supernatant, or other body fluids, such as saliva, semen, or cerebrospinal fluid, could be used to monitor the impact of the drug under development on systemic MtDNA levels in patients.

In conclusion, the growing body of evidence showing dys-regulated MtDNA levels in common diseases, both in cell free and cellular samples, support the view that MtDNA is a useful biomarker of mitochondrial dysfunction. Furthermore, emerging data from cancer, HIV and other fields indicates that MtDNA levels may correlate with patient response to treatment and are strongly suggestive that the utilisation of MtDNA as a biomarker in drug toxicity studies may be of great benefit in drug development.

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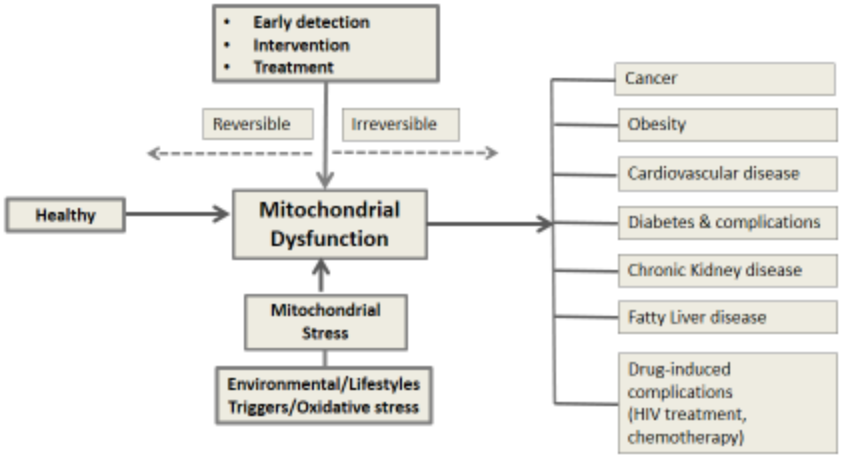

Figure 1. Mitochondrial dysfunction as an early event in disease : Environmental/Lifestyles triggers such as high fat and/or glucose or drugs can result in oxidative stress and altered signaling which in turn damages mitochondria in organs (e.g. kidney, heart, liver); cells (blood cells, adipocytes), and blood vessels, the damage may take decades to manifest itself and cause pathology. Identification of biomarkers for the early detection of the metabolic and bioenergetic changes associated with these pathologies could allow intervention and prevention of irreversible bioenergetic dysfunction.

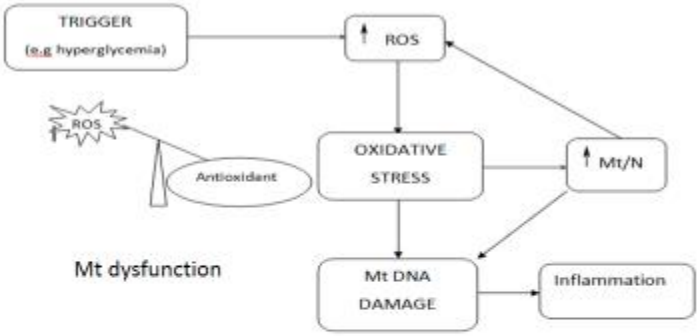

Figure 2. Schematic of the Hypothesis that Mitochondrial DNA can increase in response to oxidative stress as an adaptive response. Environmental/Lifestyles triggers such as high fat and/or glucose or drugs result in oxidative stress and altered signaling which leads to an early adaptive response of increased cellular MtDNA but over time causes systemic damage to mitochondria in organs (e.g. kidney, heart, liver) and cells (blood cells) *Taken from Malik and Czajka (2012)*

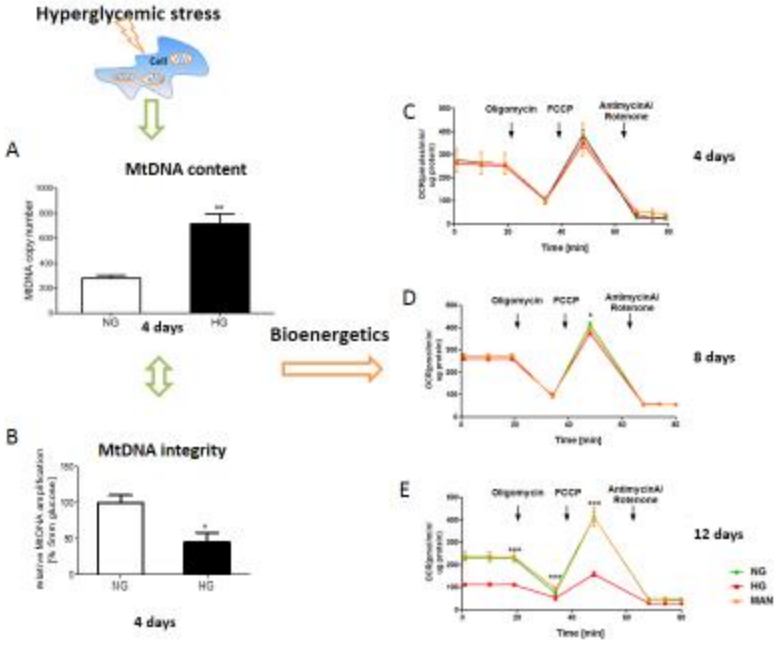

Figure 3. Changes in cellular MtDNA precede metabolic dysfunction in conditions of oxidative stress
Growth of HMCs in high glucose led to a significant increase in cellular MtDNA which was detectable within 24 hours, and highly significant after 4 days (A), however the MtDNA was damaged as illustrated by reduced amplification of an MtDNA 8.6kb fragment (B). Cells showed normal bio-energetic profile at 4 days (C), however after 8 days, maximal respiration and reserve capacity were significantly reduced in hyperglycemia cells but unaffected in normoglycmeic cells (D,E). See Czajka et al., 2015 for the full data

Table 1

	Mitochondrial DNA	Nuclear DNA
Cellular location	Mitochondria	Nucleus
Size (nt)	16569	~30,000,000,000
Major Function	DNA replication and transcription, signalling	DNA replication and transcription
Organisation	Double stranded circular molecule complexed with TFAM	Double stranded duplex linear DNA molecules (chromosomes) complexed with histones to form chromatin
Genetic code	Different use of start and stop codons	Universal
Replication	Bidirectional from a single origin of replication	Numerous origins of replication
Transcription	Poly-cistronic mRNAs from 2 promoters	Highly regulated and mostly individual mRNA transcription from thousands of individual promoters
Introns/exons	No introns, very few non coding regions, contiguous and overlapping	Contain introns and large stretches of non-coding regions
Inheritance	Maternal	Bi-parental
Replication	Independent of the cell cycle	Dependent on the cell cycle
Number of copies per cell	10s to many 1000s –variable and can change in response to physiological stimuli	2 (1 in the case of the sex chromosomes)-fixed
Methylation	Resembles bacterial DNA (less methylated)	Methylated
Sequence identity	Contains very few regions which are unique (>90% is duplicated in the nuclear genome)	Contains pseudogenes known as NUMTs which are identical to MtDNA

END